

Antibiotic Control of *Mycoplasma* in Tissue Culture

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ABSTRACT

Seven of eight strains of *Mycoplasma* (PPLO) were found to be sensitive to the deoxystreptamines, certain macrolides, and the tetracyclines. These antibiotics are relative noncytotoxic. Kanamycin and tetracycline were useful in eliminating PPLO (pleuropneumonia-like organisms) strain Squibb no. 1 from a HeLa cell line which was deliberately contaminated with PPLO. Repeated exposure of *M. laidlawii* type B cells to neomycin resulted in a 50-fold increase in resistance, and the resistant strain was also resistant to gentamicin, kanamycin, neomycin, and paromomycin. A tetracycline-resistant strain of this culture was found to be resistant to 7-chlorotetracycline, 7-chlor-6-demethyltetracycline, and 5-hydroxytetracycline. One PPLO strain, Squibb no. 2, derived from a contaminated HeLa cell culture, was resistant to all antibiotics studied.

Biochemists and microbiologists using cell cultures as biochemical systems have become increasingly aware of the problems introduced by contaminating microorganisms. Although antibiotics have been widely used to eliminate bacterial, fungal, and yeast contamination, comparatively little attention has been paid to *Mycoplasma* (PPLO) contamination. Among the methods suggested for eliminating these parasites from cell lines are heat treatment (8), use of specific antiserum (14), and antibiotic treatment. Among the antibiotics used in various laboratories were tetracycline (3, 9), kanamycin (5, 15), tylosin (6), 7-chlorotetracycline (7), and a mixture of chloramphenicol and novobiocin (2).

Although Carski and Sheppard (3) reported elimination of *Mycoplasma* from cell lines cultivated through four successive passages in the presence of only 2.5 $\mu\text{g}/\text{ml}$ of tetracycline, Hooser et al. (10) found it necessary to use 20 $\mu\text{g}/\text{ml}$. Gori and Lee (7) noted that tetracycline treatment even at cytotoxic doses for periods up to 5 months proved capable only of depressing the severity of the infection without effecting sterilization. In a similar situation, Friend et al. (6) found that several chemotherapeutic agents, including tetracycline, kanamycin, erythromycin, chloramphenicol, polymyxin, stovarsol, and iodine, frequently "cured" the infected cultures when examined immediately after treatment, only to have the pleuropneumonia-like organisms (PPLO) reappear in the subsequent passage.

It seems likely that, with continued use of "PPLO suppressive" antibiotics in tissue culture

media, there will eventually emerge "antibiotic-resistant" *Mycoplasma* strains. Strains resistant to kanamycin have already been described (2, 16), and a strain resistant to many antibiotics has been found as a tissue culture contaminant (2).

We have examined the antibiotic sensitivity of eight *Mycoplasma* strains in vitro and have used several of the most effective of these antibiotics to control *Mycoplasma* infections in cell lines. We have also studied the problem of "induced antibiotic resistance" in *Mycoplasma* strains with the objective of determining whether antibiotic cross-resistance is found in these organisms as it is found in bacteria.

MATERIALS AND METHODS

The eight *Mycoplasma* strains used in this study included: *M. laidlawii* type B; *M. arthritis* strain L₄; *M. gallisepticum* strain A-5969-TC (from two sources); *M. hominis*, strains Campo W and 39-S4; and two strains from infected HeLa tissue cultures, designated Squibb no. 1 and Squibb no. 2. Difco PPLO Enrichment Broth (without crystal violet) supplemented with yeast extract, sodium acetate, and PPLO Serum Fraction (Difco) was used for propagating the identified *Mycoplasma* species. The Squibb cultures 1 and 2 were maintained in soy-peptone broth supplemented with yeast extract, NaCl, and human serum as mentioned by Kenny and Pollock (11). Difco PPLO Agar (with PPLO Serum Fraction) was used for the antibiotic sensitivity tests for the named species, and Pollock's (11) medium (with Noble agar) was used for the Squibb cultures.

Antibiotic sensitivity tests were carried out by use of an agar dilution method. Dilutions of the antibiotic test solution in PPLO Agar were added to petri plates

and allowed to solidify. The surface of the agar was then streaked with a heavy suspension of the PPLO test strains, and the plates were incubated for about 7 days at 37 C. Growth or absence of growth was determined by microscopic inspection. Twofold dilutions of the antibiotic test solution were used in most studies; in a few instances, smaller dilutions were used to check the minimal inhibitory concentrations. Because some of the antibiotics were chemically unstable when incubated in dilute solution at 37 C, the incubation period was restricted to 7 days (with a "preliminary observation" at 4 days). This, it was felt, more closely resembled the incubation periods used with contaminated tissue cultures than did the 14-day incubation periods sometimes used with PPLO cultures.

All of the antibiotics used in this study were obtained from commercial sources, and no further purification was attempted prior to their use. Those materials which were not water-soluble were dissolved in dimethylsulfoxide before addition to media.

Experimental infections with Squibb cultures 1 and 2 were studied in a PPLO-free HeLa cell line. The infected monolayers were examined at weekly intervals for the presence of PPLO (using Pollock's technique), with medium changes being made every 3 or 4 days. Once it was established that an infection was present, antibiotics were added, and the persistence of the infection was observed over a 4-week period. Attempts to infect this tissue culture line with *M. laidlawii* type B or with *M. gallisepticum* A-5969-TC were unsuccessful. This is in agreement with the observations of Castrejon-Diez et al. (4), who found that *M. laidlawii* type A would not grow in the FL tissue culture.

RESULTS AND DISCUSSION

Antibiotic sensitivity of Mycoplasma cultures. The eight *Mycoplasma* cultures were tested for sensitivity to 40 antibiotics by use of the agar dilution test. Only Squibb culture 2 was not inhibited by any of the antibiotics. The following 20 antibiotics were essentially inactive against *Mycoplasma* in vitro (produced no inhibition of growth of test strain at 100 µg/ml in agar dilution test): amphomycin, amphotericin B, bacitracin, benzylpenicillin, candicidin A, cycloheximide, cycloserine, etruscomycin, filipin, griseofulvin, nystatin, oleandomycin, patulin, polymyxin, ristocetin, streptomycin, trichomycin, vancomycin, vernamycins A and B, and viomycin. Of these antibiotics listed as "ineffective," a few were inhibitory to some of the seven susceptible PPLO cultures, but not to others. Studies with these antibiotics were discontinued, because we were only interested in antibiotics "active" against all the PPLO cultures. The following antibiotics were quite active in inhibiting the seven susceptible PPLO cultures: carbomycin, dactinomycin, streptothricin, stendomycin, and thiostrepton. Unfortunately, however, they are too cytotoxic

(13) to be considered for use in eliminating PPLO from tissue culture.

The 15 antibiotics which were active against the seven susceptible PPLO test strains and had relatively "low" or "no" cytotoxicity are listed in Table 1. Several of these are stable (chemically) when dissolved in tissue culture media and incubated at 37 C, whereas others are rather rapidly inactivated. The useful antibiotics may be grouped on the basis of their chemistry as well as the response in our screen: (i) the deoxystreptamines: gentamicin, kanamycin, hygromycin B, neomycin, and paromomycin; (ii) the macrolides: erythromycin, spiramycin, and tylosin; (iv) the tetracyclines: 7-chlortetracycline, 6-demethyl-7-chlortetracycline, 5-hydroxytetracycline, and tetracycline. The members of the deoxystreptamine group and the macrolide group appear to have special advantages for use in eliminating PPLO contaminants from tissue cultures, because these antibiotics have high chemical stability in tissue culture media and low cytotoxicity. The tetracyclines are quite cytotoxic and, except for 6-demethyl-7-chlortetracycline, are not stable in tissue culture media at 37 C for any great length of time. In general, our results on antibiotic sensitivity of *Mycoplasma* agree with those reported by Arai et al. (1).

Elimination of Mycoplasma from tissue cultures. Kanamycin (at 200 ppm) and tetracycline (at 10 ppm) were effective in eliminating the Squibb no. 1 strain of *Mycoplasma* from the HeLa cell line. The monolayers appeared to be free from PPLO after four treatments (medium change twice a week and harvest every 7 days). No recurrence of infection was noted when antibiotic treatment was discontinued for a 4-week period. On the other hand, neither of these two antibiotics nor any other was effective in freeing the HeLa cells from Squibb no. 2. We do not advise continued exposure of the tissue culture cells to any antibiotic for fear that an antibiotic-resistant *Mycoplasma* will develop (see below). (Note added in proof: further study has shown PPLO strain 2 to be sensitive to lincomycin at 20 ppm.)

Induced antibiotic resistance in Mycoplasma. The strain of *M. laidlawii* type B was initially found to be quite sensitive to kanamycin and neomycin (minimal inhibitory concentrations of the order of 10 µg/ml when the agar dilution test was used). When this culture was exposed to increasing concentrations of neomycin, resistant strains appeared. After eight exposures, a strain resistant to 500 µg/ml was obtained. This strain was found to be resistant to kanamycin, gentamicin, and paromomycin, though increase in resistance was not as marked (50-fold) as with neomycin. When tetracycline was used as the

TABLE 1. Antibiotics useful in controlling PPLO contamination in tissue cultures

Antibiotic	Stability in tissue culture media ^a	Concn showing marked cytotoxicity ^b	Minimal concn inhibiting PPLO in agar streak test ^c	Concn recommended for controlling PPLO in tissue cultures ^d
		$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
Chloramphenicol	High	30	15	30
7-Chlortetracycline	Very low	80	40	100
6-Demethyl-7-chlortetracycline	High	15	5	10
Erythromycin	Moderate	300	15	50
Fusidic acid	High	40	20	20
Gentamicin	High	3,000	1	200
5-Hydroxytetracycline	Moderate	35	5	10
Hygromycin B	Moderate	300	15	50
Kanamycin	Very high	10,000	25	200
Neomycin B	Very high	3,000	15	50
Novobiocin	Low	200	10	50
Paromomycin	High	5,000	20	50
Spiramycin	Moderate	1,000	1	50
Tetracycline	Moderate	35	2	10
Tylosin	Moderate	300	1	10

^a Stability scale: half-life of 2 days, very low; half-life of 4 days, low to moderate; half-life of 8 days, very high.

^b Data from Perlman and Brindle (12).

^c As determined in twofold agar dilution test (see text).

^d Recommended on basis of 3-day incubation period between medium changes.

antibiotic, a 40-fold increase in resistance was observed after seven passages. This tetracycline-resistant culture was considerably more resistant to 7-chlortetracycline and 5-hydroxytetracycline than was the parent culture. A 10-fold increase in resistance to chloramphenicol was also noted. The ease with which resistant cultures were obtained bodes ill for those who plan routinely to use certain antibiotics to eliminate *Mycoplasma* from their cell lines.

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